DIFFERENCE IN ANTIGENIC REACTIVITY AND ULTRASTRUCTURE BETWEEN FLUID-PHASE C5b-9 AND THE C5b-9 MEMBRANE ATTACK COMPLEX OF HUMAN COMPLEMENT

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1. Introduction

Complement activation on a target membrane leads to assembly of an amphiphilic, cylindrical C5b-9 protein complex, whose apolar surfaces penetrate into the lipid bilayer, causing formation of a trans-membrane pore [1–6]. By contrast, complement activation in aqueous environment (e.g., in serum) leads to the formation of a C5b-9 complex which is hydrophilic and which no longer exerts membrane-lytic activity [7,8]. This 'fluid-phase' complex, isolated and characterized [9,10], has been subsequently referred to as SC5b-9 to denote the presence of an additional protein component (S-protein) [11–13]. Data has been forwarded that this protein binds to apolar surfaces of nascent C5b-7 molecules, thereby inhibiting the potential of the trimolecular complexes to bind lipid and attach themselves to bystander membranes [11–13]. This paper provides supplementary immunochemical and ultrastructural evidence that the ensuing fluid-phase C5b-9 complex (SC5b-9) differs in molecular conformation from the C5b-9 membrane attack complex of complement (C5b-9)).

2. Materials and methods

Inulin (p.a.) was purchased from Serva, Heidelberg.

Abbreviations: SC5b-9, fluid-phase C5b-9; C5b-9(m), membrane C5b-9

Sources of antibodies and other reagents were as in [3–5,14,15].

2.1. *Isolation of SC5b-9*

Fresh human serum was treated with inulin as in [9] and SC5b-9 isolated from inulin-activated serum following a protocol similar to that in [9,16]. The isolation procedure consisted of

(i) Chromatography of inulin-activated serum over Sepharose 6B in a 10 mM Tris, 50 mM NaCl buffer (pH 8.2) containing 15 mM NaN_3; SC5b-9 elutes in a region comparable to that of IgM [9];
(ii) Centrifugation of the concentrated fractions containing SC5b-9 over linear 25–50% sucrose density gradients in the same buffer system; SC5b-9 sediments as a 22.5 S protein and separates from the bulk of contaminating proteins [16];
(iii) Preparative agarose gel electrophoresis using line electrophoresis [17] in horizontal agarose slabs (10 × 20 × 0.5 cm); SC5b-9 separates by virtue of its α-electrophoretic mobility at pH 8.7 from other proteins of low electrophoretic mobility [9]. Agarose strips containing SC5b-9 were eluted electrophoretically as in [15];
(iv) Two recentrifugations over linear 25–50% sucrose density gradients.

SC5b-9 was detected in fused rocket immunoelectrophoresis [18] and crossed immunoelectrophoresis after each isolation step by using the cross-reacting antiserum to membrane C5b-9 [3,4]. The isolated protein exhibited the following properties:

(i) It yielded an immunoprecipitate in crossed immunoelectrophoresis and double-diffusion when tested with an antiserum specific for membrane C5b-9; in double-diffusion, this immunoprecipitate coalesced fully with that of membrane C5b-9;
(ii) It gave rise to a corresponding immunoprecipitate when tested with a polyspecific antiserum in crossed immunoelectrophoresis; in addition, two minor serum protein contaminants were detected with this antiserum.
(iii) Its sedimentation coefficient was $s^{\circ}_{20,\,solv} = 22.3$ S as determined by analytical ultracentrifugation. The concentration dependence of the sedimentation rate was 0.24 S/mg/ml.
(iv) It possessed α-electrophoretic mobility in agarose at pH 8.7.
(v) It reacted with specific anti-C5, anti-C6 and anti-C9 in double-diffusion to yield coalescing immunoprecipitates (in accordance with [9]).

Purified SC5b-9 (1.0–1.5 mg) were recovered from 100 ml of inulin-inactivated serum. Recoveries were in the order of 10–15% of total SC5b-9 present in inulin-activated serum, as estimated by quantitative immunoelectrophoresis [19]. The concentration of SC5b-9 in inulin-activated serum was thus estimated to be 150–200 μg/ml.

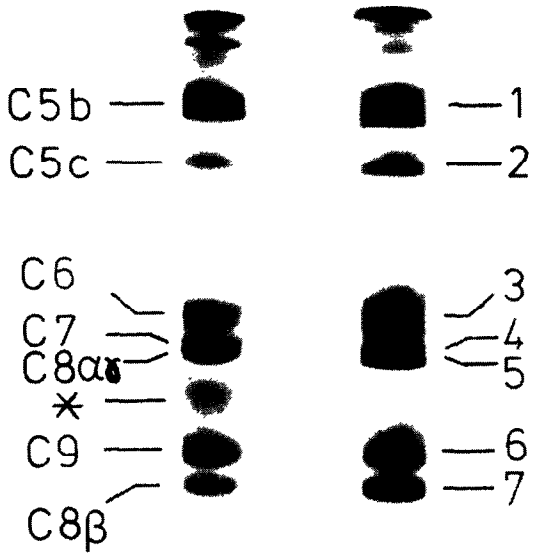

Fig.1. SDS–polyacrylamide gel electrophoresis of SC5b-9 (left) and C5b-9(m) (right). Complement components are labelled according to [9,16] (left) and numbered as in [15] (right). Asterisk marks the additional protein band of SC5b-9 corresponding to the S-protein [11–13], which is not found in the electrophoresis pattern of C5b-9(m).

2.2. *Analytical methods*

SDS–polyacrylamide gel electrophoresis, quantitative immunoelectrophoresis, and electron microscopy were as in [3,14,15]. Charge-shift crossed immunoelectrophoresis and autoradiographical analyses of Triton X-100 binding in crossed immunoelectrophoresis were performed as in [20,21]. Analytical ultracentrifugations were kindly performed by Dr B. Kickhöfen and H. Kochanowski (Max-Planck-Institut für Immunbiologie, Freiburg) employing a Beckmann Model E analytical ultracentrifuge equipped with a Schlieren optical system, temperature control unit (RTIC) and electronic speed control. The rotor operated at 34 000 rev./min and 20°C. Sedimentation velocity experiments were performed at three protein concentrations in 10 mM Tris, 50 mM NaCl (pH 8.2).

3. Results and discussion

3.1. *SDS–polyacrylamide gel electrophoresis patterns of SC5b-9 and C5b-9(m)*

The SDS–polyacrylamide gel electrophoresis pattern of SC5b-9 and C5b-9(m) are shown in fig.1. The major bands of both preparations, designated and numbered as in [9,15,16] match each other and are in agreement with the data in [9,15]. The apparent molecular weights of the individual proteins have been given in [9,15]. The protein band marked by an asterisk in the pattern of SC5b-9 has never been found in preparations of C5b-9(m), and corresponds positionally to the additional protein band in [9],

later referred to as the S-protein [11–13]. The present findings confirm the presence of this protein in preparations of SC5b-9. Its concentration in relation to the C5-C9 protein bands has been found to vary somewhat in different preparations. This protein has been suggested to function as an inhibitor of nascent C5b-7, covering the lipid-binding sites of the trimolecular complex [11–13]. It has also been reported that chromatography of deoxycholate-treated SC5b-9 in this detergent selectively removes the S-protein from the complex, rendering the complex amphiphilic [13]. For reasons unclear at present, we have not succeeded in removing the S-protein from SC5b-9 using deoxycholate. The cause of this discrepancy is being investigated.

3.2. *Hydrophilic nature of SC5b-9*

SC5b-9 behaved like a hydrophilic protein in charge-shift electrophoresis [20,22], exhibiting a unidirectional charge-shift in deoxycholate but no shift in cetyltrimethylammoniumbromide (not shown). It also did not bind Triton X-100, as judged by an autoradiographical analysis using crossed immunoelectrophoresis (not shown). These data are consistent with the finding that SC5b-9 is lytically inactive [7,8].

3.3. *Reaction of SC5b-9 with an antiserum to C5b-9(m)*

A difference in antigenic reactivity of SC5b-9 compared to its membrane counterpart was detected using an antiserum to C5b-9(m)-neoantigens. Figure 2A shows a rocket immunoelectrophoresis of C5b-9(m) (left well) and SC5b-9 (right well) using an antiserum to C5b-9(m). Samples were applied such that identical rocket heights were obtained. Figure 2B shows the result of an analogous rocket immunoelectrophoresis using identical samples but an antiserum to C5b-9(m)-neoantigens. Whereas C5b-9(m)

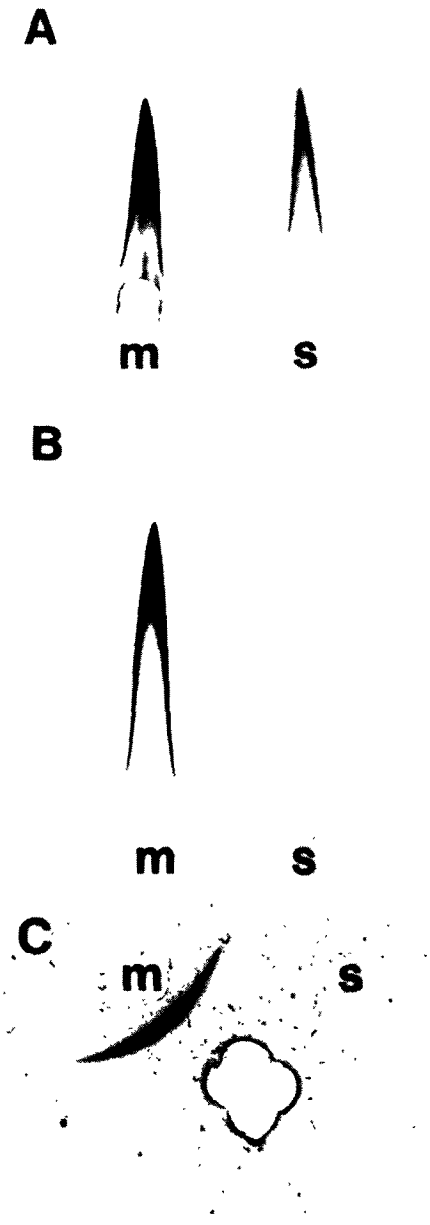

Fig.2. Difference in antigenic reactivity between SC5b-9 and C5b-9(m). 2A: C5b-9(m) (left, (m)) and SC5b-9 (right, (s)) were subjected to rocket immunoelectrophoresis using anti-C5b-9(m). Note identical rocket heights. In fig.2B identical samples were applied using an antiserum to C5b-9(m)-neoantigens [2]. The weak reaction of SC5b-9 indicates that not all neoantigens of the membrane complex are present on the surface of SC5b-9. 2C: double-diffusion analysis of C5b-9(m) (left well) and SC5b-9 (right well) using anti-C5b-9(m) which had been absorbed with an excess of unfractionated, inulin-activated serum in the presence of 10 mM EDTA. The absorbed antiserum still precipitates the membrane complex but no longer precipitates SC5b-9. Identical results were obtained using gels with and without Triton X-100.

is well precipitated by this antiserum, SC5b-9 reacts only weakly, yielding a faint and higher immunoprecipitate. When an antiserum to C5b-9(m) was absorbed with an excess of inulin-activated serum in the presence of EDTA, the resulting antiserum precipitated the membrane complex, but no longer precipitated SC5b-9 (fig.2C). Thus, fewer neoantigenic determinants characteristic of C5b-9(m) are exposed on the surface of SC5b-9.

3.4. *Ultrastructure of SC5b-9*

Electron microscopy on negative stainings of purified SC5b-9 preparations revealed uniformly dispersed, globular units with indistinct, irregular contours of ~20 nm diam. The globules exhibited inhomogeneous electron density, suggesting the presence of cavities in the molecule (fig.3A,3B). A few appeared as fuzzy ring structures and rectangles, containing central stain deposits. The dimensions of such structures are comparable to axial and side projections of the C5b-9(m) molecule, which is shown for comparison in fig.3C. The latter complexes are, however, distinctly contoured, and also exhibit a pronounced tendency to aggregate.

The ultrastructural findings provide direct evidence for a difference in molecular conformation of SC5b-9 as opposed to C5b-9(m). Profiles reminiscent of the cylindrical C5b-9(m) molecule occur sporadically in

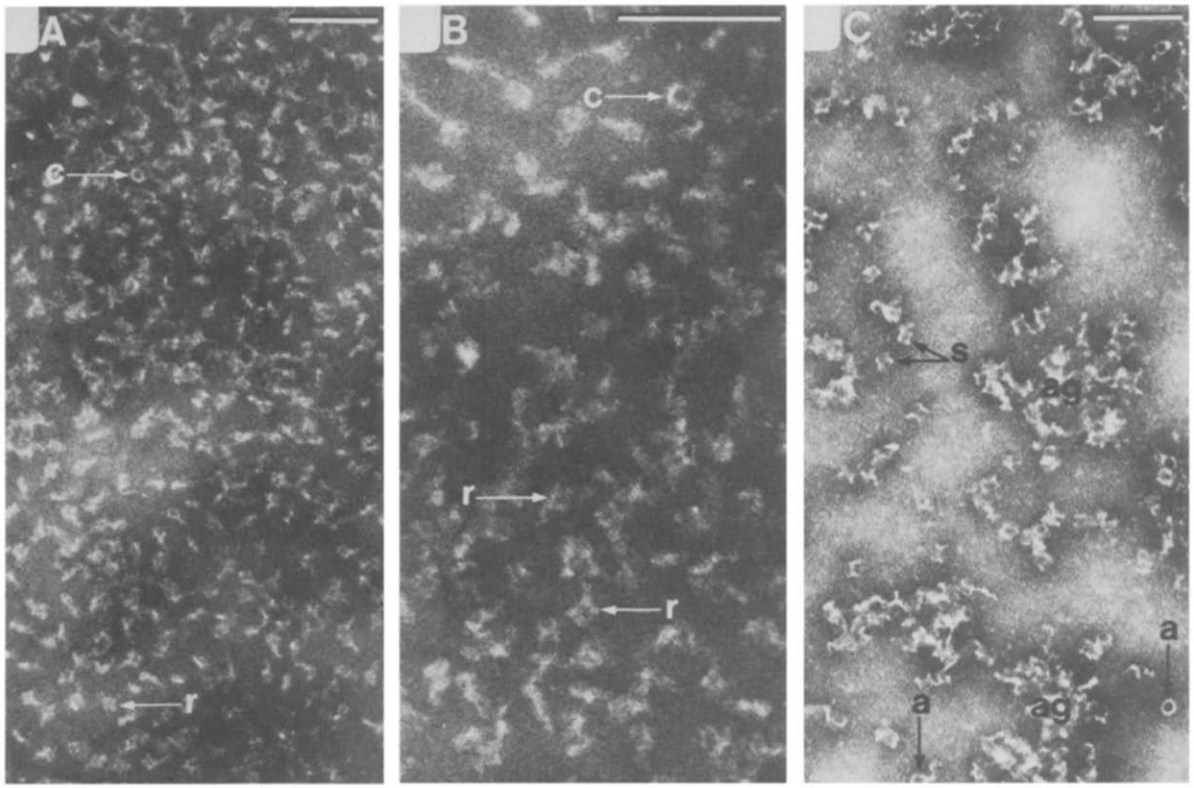

Fig.3. Electron micrographs of purified SC5b-9, negatively stained with sodium silicotungstate. The complexes are seen as uniformly dispersed, irregular and fuzzy globules. Some of the globules are seen as indistinct, rectangular profiles (r) containing a central stain deposit. This, together with the sporadic observation of ring-shaped profiles (c), is reminiscent of the side (s) and axial (a) projections of the cylindrical C5b-9(m) molecules. Figure 3C depicts a similar negative staining of a native C5b-9(m) preparation which is shown for comparison. Apart from the clearly unidentical ultrastructures of the complexes, the native membrane complex also exhibits a marked tendency to aggregate (ag) even in the presence of Triton X-100 + deoxycholate. Scale bars indicate 100 nm.

the preparations, and this observation may point to a cylindrical structure inherent, but masked, in the SC5b-9 molecule as well.

The absence of aggregate formation by SC5b-9 as judged by electron microscopy is in accordance with the known hydrophilic nature of SC5b-9 [7,8,13], and contrasts with the pronounced aggregation tendency exhibited by the amphiphilic C5b-9(m) complexes [3].

The immunochemical observations as well as the ultrastructural data could both be brought into accordance with the concept that the S-protein binds to and conceals molecular surfaces of SC5b-9. Additionally, the hydrophilic environment in which the complex is generated may also directly influence its conformation, leading to concealment of the apolar molecular surfaces within the complex and to the observed alteration in antigenic reactivity and ultrastructure of SC5b-9 as opposed to that of the C5b-9(m) membrane attack complex of complement.

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